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# Investigation of Specific Substitutions in Virulence Genes Characterizing Phenotypic Groups of Low-Virulence Field Strains of *Listeria monocytogenes*

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**Several models have shown that virulence varies from one strain of *Listeria monocytogenes* to another, but little is known about the cause of low virulence. Twenty-six field *L. monocytogenes* strains were shown to be of low virulence in a plaque-forming assay and in a subcutaneous inoculation test in mice. Using the results of cell infection assays and phospholipase activities, the low-virulence strains were assigned to one of four groups by cluster analysis and then virulence-related genes were sequenced. Group I included 11 strains that did not enter cells and had no phospholipase activity. These strains exhibited a mutated PrfA; eight strains had a single amino acid substitution, PrfAK220T, and the other three had a truncated PrfA, PrfAΔ174-237. These genetic modifications could explain the low virulence of group I strains, since mutated PrfA proteins were inactive. Group II and III strains entered cells but did not form plaques. Group II strains had low phosphatidylcholine phospholipase C activity, whereas group III strains had low phosphatidylinositol phospholipase C activity. Several substitutions were observed for five out of six group III strains in the *plcA* gene and for one out of three group II strains in the *plcB* gene. Group IV strains poorly colonized spleens of mice and were practically indistinguishable from fully virulent strains on the basis of the above-mentioned in vitro criteria. These results demonstrate a relationship between the phenotypic classification and the genotypic modifications for at least group I and III strains and suggest a common evolution of these strains within a group.**

*Listeria monocytogenes*, a facultative intracellular pathogen, is a major cause of food-borne infection in humans (9, 40). Although rare, invasive listeriosis is a public health concern because of its severity, with a fatality rate evaluated at 20 to 30%, and possible sequelae because of its potential to cause epidemics (34). Current surveillance schemes assume that all the isolates of *L. monocytogenes* are equally pathogenic, but several observations have suggested that virulence varies from one strain to another. This variation has been demonstrated in studies of bacterial surface immunodeterminants in clinical and food strains, as well as in studies of infection in animals (2, 20, 25, 30). For example, 8% to 21% of field *L. monocytogenes* strains are nonvirulent or weakly virulent in mice or cell monolayer assays (7, 31, 38, 42). There are few explanations for the low virulence of these strains: a few of them do not produce hemolysin (8), and some have truncated internalin (19).

The first step in infection is adhesion to the eukaryotic cells via bacterial surface factors such as adhesins (14) and the Ami protein (29). In addition, several bacterial factors, including two proteins of the internalin family, InlA (or internalin) and InlB (12), facilitate entry of *L. monocytogenes* into the host cell, where it lies in a phagocytic vacuole. The vacuole is quickly lysed by the pore-forming toxin listeriolysin O (LLO), encoded by the *hly* gene, and the two phospholipases C phosphatidylinositol phospholipase C (PI-PLC) and phosphatidylcholine phospholipase C (PC-PLC), encoded by the *plcA* and *plcB* genes, respectively (5, 26, 44). The free *L. monocytogenes* in the cytosol replicates and induces polymerization of actin filaments that promote intracellular bacterial movement (39), allowing the formation of a bacterium-containing protrusion. The protrusion contacts a neighboring cell, enters it, and gives rise to a two-membrane vacuole lysed by PC-PLC and LLO (44). Thus, the bacterium is disseminated by direct cell-to-cell contact. This enables formation of plaques in cell monolayers (23), a property that has been correlated with virulence for mice (42). Most of these genes are found in a 10-kb cluster which also encodes the main virulence regulator PrfA.

We previously identified 26 low-virulence *L. monocytogenes* strains by using a method that combines a plaque-forming (PF)

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assay with the subcutaneous (s.c.) inoculation of mice (33). These strains exhibit a low lethality in mice, and their full virulence could not be restored after 10 successive inoculations (32). The present study was designed to identify virulence factors or cell invasion mechanisms that had been altered in these low-virulence strains.

## MATERIALS AND METHODS

**Strains and culture conditions.** The 40 *L. monocytogenes* strains and 1 *Listeria innocua* strain used in this study are described in Table 1. All isolates examined were from independent sources and had different isolation dates. The *L. monocytogenes* strains were defined as virulent or low-virulence strains by a virulence test combining a PF assay and s.c. inoculation of mice (33). In each assay low-virulence strains were compared to virulent strains. Our PCR studies showed that they possessed the virulence genes *prfA*, *hly*, *plcA*, *plcB*, *mpl*, and *actA* (data not shown). The strains were maintained in storage medium (Sanofi Pasteur, Bio-Rad, Ivry-sur-Seine, France) at 4°C. For analysis, they were cultured for 8 h in brain heart infusion (BHI) (Difco, Becton Dickinson, Meylan, France) broth at 37°C.

The 26 low-virulence strains were always compared to a single *L. innocua* strain and 13 virulent strains whose virulence had been confirmed by PF assays, 50% lethal doses, and spleen colonization after intravenous and s.c. injection (31).

All transformant strains were grown in the presence of erythromycin (Sigma, Saint-Quentin Fallavier, France) at a final concentration of 1 mg $ml^{-1}$ on tryptic soy agar (TSA) (Biomérieux, Marcy L'Etoile, France). The final concentrations of erythromycin in liquid media were 150 μg $ml^{-1}$ for *Escherichia coli* MC1061 (6) and 8 μg $ml^{-1}$ for *L. monocytogenes* strains.

**Cell line and culture conditions.** Cells of the human adenocarcinoma line HT-29 (no. 85061109; ECACC, Salisbury, United Kingdom) (11), used between passages 24 and 66, were grown in 75-$cm^2$ plastic tissue culture flasks (Nunc, Invitrogen, Cergy Pontoise, France) in Dulbecco's modified Eagle's medium with glucose (4.5 g/liter) (Invitrogen) supplemented with 10% (vol/vol) fetal calf serum (Invitrogen) and 2 mM L-glutamine (Invitrogen). Antibiotics (100 IU penicillin per ml and 100 μg streptomycin per ml) were routinely added to the culture medium except for the virulence assays. Cells were maintained in a humidified incubator at 37°C under 5% (vol/vol) $CO_2$.

**Cell assays.** The different steps of the cell infections were analyzed with HT-29 cells grown on 24-well tissue culture plates (Falcon) for 5 days to obtain almost confluent monolayers. Each experiment used two plates: one for the adhesion-invasion of bacteria and the second for the invasion. HT-29 cell monolayers were incubated in medium without antibiotics for 24 h and then infected for 2 h at 37°C with $10^7$ CFU in 300 μl (multiplicity of infection = 5). For adhesion-invasion assays, the cell monolayers were gently washed six times with phosphate-buffered saline (pH 7.3) and then disrupted with 1 ml cold distilled water (4°C). Viable bacteria (intra- and extracellular) were counted after plating serial dilutions on TSA. The other plates were washed with Dulbecco's modified Eagle's medium and incubated in culture medium containing 100 μg of gentamicin per ml. After 1.5 h at 37°C, cells were washed with phosphate-buffered saline and lysed with 1 ml cold distilled water (4°C). Viable intracellular bacteria were assessed by serial dilutions plated on TSA. Monolayer integrity and cell viability were checked in parallel to infection by the trypan blue exclusion test (41). The results were expressed as the percentage of CFU recovered after 2 h (adhesion-invasion) and 3.5 h (invasion) relative to the number of bacteria deposited per well. Experiments were carried out in duplicate and repeated twice for each strain.

The virulence of the strains transformed with a plasmid carrying the *prfA* open reading frame (ORF) was assessed in a PF assay with HT-29 cells (33). All inocula of transformant *L. monocytogenes* strains were prepared in the presence of erythromycin.

**Titration of phospholipase C activities.** The PI-PLC activity was assessed essentially as described by Goldfine and Knob (15). An overnight culture of bacteria at 37°C in BHI broth was diluted 10-fold in 9 ml of BHI broth supplemented with 0.2% activated charcoal (Prolabo, Merck, Strasbourg, France) (BHI-AC) and incubated at 37°C on a rotary shaker at 150 rpm for 7 h. A 1-ml aliquot of exponentially growing bacterial suspension was removed and filtered (0.2-μm filter; Sartorius, Palaiseau, France). One hundred microliters of filtrate was incubated for 10 min at 37°C with 100 μl L-α-phosphatidylinositol {L-α-[*myo*-inositol-2-$^3$H(N)]; NEN Life Science Products, Le Blanc Mesnil, France} (0.9 μCi $ml^{-1}$; 18.5 Ci $mmol^{-1}$) and 13.5 μg of L-α-phosphatidylinositol (Sigma) that had been sonicated and diluted in 40 mM Tris-HCl (pH 7.2)–0.2% deoxycholate. The reaction was stopped by adding 200 μl chloroform–methanol (1:1 [vol/vol]) and 100 μl 0.1 N HCl. The mixture was centrifuged for 7 min at 225 × *g*, and the water-soluble [$^3$H]inositol phosphate was measured in a liquid scintillation analyzer (Packard 1600 TR); 1 unit of activity represents the release of 1 μmol inositol phosphate per min. Quantification of the number of CFU was carried out on TSA plates to check that the strains could be compared. Experiments were performed in duplicate and repeated twice for each strain.

The PC-PLC activity was assessed essentially as described by Geoffroy et al. (13). BHI-AC broth was seeded after preculture in BHI broth and incubated overnight at 37°C. The stationary-growth-phase bacterial suspension was filtered as for PI-PLC titration. The PC-PLC activity was assessed by incubating 100 μl filtrate for 24 h at 37°C in 900 μl lecithin suspension, made by adding 200 μl lecithin solution (3.6% lecithin [Sigma], 2.4% sodium cholate [Sigma], 1 mM $ZnSO_4$ [Sigma], 0.1% sodium azide [Sigma], 100 ml distilled water) to 700 μl 0.15 M NaCl (Carlo Erba). The PC released was measured at 510 nm by comparison with a reference suspension (100 μl sterile BHI-AC filtrate in 900 μl lecithin suspension). PC-PLC units are defined as the amount of enzyme increasing the optical density at 510 nm by 0.1 unit in 1 h. Experiments were performed in duplicate and repeated twice for each strain. Quantification of the number of CFU was carried out on TSA plates to check that the strains could be compared.

**Titration of hemolytic activity.** Hemolytic activity was assessed as described by Roche et al. (33). One hemolytic unit is defined as the reciprocal of the dilution at which 50% hemolysis is detected. Experiments were carried out in duplicate and repeated twice for each strain.

**Statistical analysis.** A model-based agglomerative hierarchical clustering analysis was performed to look for groups (clusters) in the data, in such a way that objects belonging to the same cluster resembled each other, whereas objects in different clusters were dissimilar. A hierarchical algorithm was used, as it yielded a complete hierarchy of clustering for the given data set. We applied the most widely used ascendant clustering hierarchical technique. This model-based agglomerative hierarchical clustering is based on the assumption that the data are generated by a mixture of underlying probability distributions (1). It is assumed that the population of interest consists of different subpopulations, each with its own probability distribution. A wide range of probability distributions can be assumed. A maximum-likelihood classification is performed to determine the different subpopulations (clusters). Ward's method, based on a sum-of-squares criterion, is one example of this approach.

We used an S* criterion with the commercial software S-Plus for Windows (1, 18), which allows ellipsoidal distributions with different variances to ensure the flexibility of the classification, as we suspected the presence of a group with characteristics similar to those of the virulent strains with regard to the cell infection phenotype and phospholipase C activities. This group had a greater internal variance than the others.

**Nucleotide sequencing of *prfA*, *plcA*, and *plcB* genes and sequence analyses.** The DNAs of the *L. monocytogenes* strains were amplified by PCR from total DNA (28), with DyNAzym DNA polymerase (Finnzymes, Ozyme, Saint-Quentin en Yvelines, France) for the *prfA* genes or with *Pfu* polymerase (Promega, Charbonnières, France) for the *plcA* and *plcB* genes, for 35 cycles in a thermal cycler (iCycler; Bio-Rad, Marnes La Coquette, France). Denaturation and elongation steps were performed for 30 s at 95°C and 90 s at 72°C, respectively, except for the *plcA* gene, for which elongation was performed for 3 min. The hybridization step was carried out for 45 s at 55°C for the *prfA* genes and for 30 s at 55°C for the *plcA* and *plcB* genes. External primers located in genes surrounding them are described in Table 2. Nucleotide sequencing of the *prfA* genes was carried out using Big Dye terminator sequencing chemistry (Applied Biosystems, Waters, Saint-Quentin en Yvelines, France) and an automated 3700 DNA sequencer (Perkin-Elmer, NEN Life Science Products, Le Blanc-Mesnil, France). Nucleotide sequences were aligned using Vector NTI (Informax, Invitrogen) and protein sequences using Clustal W. Nucleotide sequencing of the *plcA* and *plcB* genes was carried out by Genome Express (Genome Express, Meylan, France). Nucleotide and protein sequences were aligned using Vector NTI.

**Constructions of plasmids and strains for *prfA trans*-complementation.** Two complementation experiments were carried out. First, the EGD Δ*prfA* strain was transformed with the wild-type *prfA* gene from the EGDe strain or with mutated *prfA* genes from four low-virulence strains (BO18, AF95, CNL895806, and SO49). Second, these four low-virulence strains were transformed with the wild-type *prfA* gene of the EGDe strain.

The pP1 vector (10), carrying a strong constitutive promoter of the protease of *Streptococcus cremoris*, was used to clone and express the *prfA* ORF of each strain. All ORFs with the transcriptional terminator of the *prfA* gene were amplified by PCR with *Pfu* DNA polymerase (Promega) for 35 cycles of 30 s at 95°C, 45 s at 54°C, and 110 s at 72°C. We used primer A (5′GGTCTAGAC GATTGGGGGATGAGAC3′ [XbaI site underlined]) and primer B (5′GGG TCGACCAGCTCTTCTTGGTGAAG3′ [SalI site underlined]). PCR fragments

TABLE 1. Characteristics of *Listeria* strains used

| *Listeria* strains[a] | Source | Serovar | Virulence (mean ± SD) by: | | |
|---|---|---|---|---|---|
| | | | PF assay[b] | s.c. test (spleens)[c] | *I/T*[d] |
| *L. monocytogenes* | | | | | |
| EGDe Δ*prfA*[h] | | | 0 | 0 | 0/5 |
| Virulent strains | | | | | |
| AF28[e] | Food product | 4b | 6.60 ± 0.03 | 5.83 ± 0.33 | 5/5 |
| EGDe BUG1600[e] | Animal clinical case | 1/2a | 6.35 ± 0.12 | 5.54 ± 0.46 | 5/5 |
| SO93[e] | Food-manufacturing plant | 1/2b | 6.00 ± 0.12 | 4.78 ± 0.71 | 5/5 |
| LO28[f] | Human isolate | 1/2c | 5.21 ± 0.16 | 4.66 ± 0.29 | 5/5 |
| SO131[e] | Food product | 1/2c | 4.76 ± 0.39 | 5.12 ± 0.43 | 5/5 |
| SO55[e] | Food product | 4b | 4.72 ± 0.45 | 3.97 ± 0.41 | 5/5 |
| SO17[e] | Food-manufacturing plant | 4b | 4.66 ± 0.11 | 4.52 ± 0.36 | 5/5 |
| AF31[e] | Food product | 1/2a | 4.56 ± 0.33 | 5.46 ± 0.32 | 5/5 |
| CR990[g] | Human clinical case | 4b | 4.07 ± 0.31 | 3.18 ± 0.29 | 4/5 |
| SO154[e] | Food-manufacturing plant | 1/2c | 3.99 ± 0.31 | 6.25 ± 0.48 | 5/5 |
| 446[f] | Food product | 4b | 3.70 ± 0.16 | 3.95 ± 0.41 | 4/5 |
| CR015[g] | Human clinical case | 1/2a | 3.06 ± 0.03 | 4.45 ± 0.75 | 5/5 |
| SO223[e] | Food-manufacturing plant | 1/2a | 2.19 ± 0.24 | 5.62 ± 0.19 | 5/5 |
| Low-virulence strains | | | | | |
| Group IV strains | | | | | |
| 436[f] | Food product | 4b | 6.31 ± 0.30 | 2.81 ± 0.68 | 12/20 |
| 464[f] | Human clinical case | 4b | 5.70 ± 0.22 | 2.59 ± 0.39 | 9/15 |
| BO34[e] | Food-manufacturing plant | 4d | 3.28 ± 0.22 | 3.63 ± 0.56 | 5/10 |
| CR282[g] | Human clinical case | 7 | 3.10 ± 0.10 | 3.01 ± 0.61 | 2/10 |
| 449[f] | Food product | 4b | 2.98 ± 0.40 | 0 | 0/5 |
| 442[f] | Food product | 4d or e | 2.58 ± 0.11 | 0 | 0/10 |
| Group III strains | | | | | |
| 417[f] | Food product | 1/2a | 0 | 2.81 ± 1.47 | 2/20 |
| AF105[e] | Food product | 1/2c | 0 | 0 | 0/5 |
| CNL895807[f] | Food product | 1/2a | 0 | 3 | 1/25 |
| 416[f] | Food product | 1/2a | 0 | 0 | 0/5 |
| CNL895795[f] | Food product | 1/2a | 0 | 0 | 0/5 |
| BO43[e] | Food product | 1/2a | 0 | 2.53 | 1/5 |
| Group II strains | | | | | |
| 454[f] | Food product | 4d/e | 0 | 3.26 ± 0.53 | 3/20 |
| SO207[e] | Food-manufacturing plant | 1/2c | 0 | 0 | 0/5 |
| SO205[e] | Food-manufacturing plant | 1/2c | 0 | 2.82 ± 0.33 | 2/10 |
| Group I strains | | | | | |
| AF95[e] | Food product | 1/2a | 0 | 0 | 0/5 |
| CNL895806[f] | Food product | 1/2a | 0 | 0 | 0/5 |
| AF10[e] | Food product | 1/2a | 0 | 0 | 0/5 |
| BO38[f] | Food-manufacturing plant | 1/2a | 0 | 0 | 0/5 |
| CNL895803[f] | Food product | 1/2a | 0 | 0 | 0/5 |
| CHU860776[f] | Food product | 1/2a | 0 | 0 | 0/5 |
| BO18[e] | Food product | 1/2a | 0 | 1.31 | 1/10 |
| CNL895793[f] | Food product | 1/2a | 0 | 0 | 0/5 |
| CNL895804[f] | Food product | 1/2a | 0 | 0 | 0/5 |
| CNL895809[f] | Food product | 1/2a | 0 | 0 | 0/5 |
| SO49[e] | Food product | 1/2a | 0 | 0 | 0/5 |
| *L. innocua* BUG499[g] | | 6a | 0 | 0 | 0/5 |

[a] The strains came from Soredab (La Boissière-Ecole, France) (SO and BO strains), Agence Française de Sécurité Sanitaire des Aliments (Maisons-Alfort, France) (AF strains), the laboratory of P. Cossart (Institut Pasteur, Paris, France) (BUG strains), Centre National de Référence des *Listeria* (Institut Pasteur, Paris, France) (CR strains), and the laboratory of P. Berche (Faculté de Médecine Necker-Enfants Malades, Paris, France) (strain LO28). Other strains came from the laboratory of A. Audurier (Université de Tours, Unité EA 2105, Tours, France). All isolates were isolated from independent sources.

[b] Log numbers of plaques per $10^7$ CFU deposited. Values are from two independent experiments performed in duplicate.

[c] Log numbers of *Listeria* recovered in the spleens 3 days after s.c. injection into the left hind footpads of immunocompetent Swiss mice with $10^4$ CFU in 50 μl. Values are from infected mice.

[d] Ratio of infected mice to inoculated mice in s.c. test.

[e] Strain previously described by Gracieux et al. (16).

[f] Strain previously described by Roche et al. (33).

[g] Strain previously described by Roche et al. (3).

[h] EGD strain with the *prfA* gene deleted, as described by Bockmann et al. (4).

TABLE 2. Primers used in this study

| Gene | Primer sequence (5′ to 3′) | Product length (bp) |
|---|---|---|
| *prfA* | Forward, GGGGTACCCCTCGTACTCAACTTAACATC<br>Reverse, GCTCTAGAGCAAACTCCATCGCTCTTCCAG | 1285 |
| *plcA* | Forward, CTCGTGAGCTTTGTGATACC<br>Reverse, GATTGGCGTCTTAGGACTTGCAGG | 1773 |
| *plcB* | Forward, ATTGGCGTGTTCTCTTTAGG<br>Reverse, TTAATACGGAACATAACGCG | 1103 |

were digested with the restriction enzymes SalI and XbaI, which were purchased from Promega and used as recommended by the manufacturer. The fragments were ligated with a DNA ligation kit (Takara, BioWhittaker, Emerainville, France) into SalI-XbaI-digested pP1 vector. $CaCl_2$ treatment was used to introduce the recombinant plasmids into *E. coli* MC1061 (17). Transformant strains were selected by antibiotic resistance, and the integration of the *prfA* ORF into pP1 was confirmed by isolation of plasmid DNA and restriction analysis (see above) (3). The integrity of the insert for each construction was checked by sequencing both strands (Genome Express, Meylan, France). EGD Δ*prfA* or low-virulence strains were then transformed by electroporation as described by Sheehan et al. (36). Transformant strains were selected by their antibiotic resistance and by their specific restriction enzyme patterns.

**Mouse virulence assays.** The virulence of the strains transformed with plasmid harboring the *prfA* ORF was assessed after subcutaneous injection into the left hind footpads of 7-week-old conventional Swiss female mice (Iffa-Credo, L'Arbresle, France) (33). All the inocula of transformant *L. monocytogenes* strains were prepared in the presence of erythromycin. In brief, 4 log CFU were injected subcutaneously into the left hind footpad. Five mice were injected per strain. They were maintained under a controlled atmosphere during the experiments and sacrificed by cervical dislocation 3 days after s.c. inoculation. Spleens were removed and soon after were homogenized. Dilutions were performed, and viable bacteria were assessed on both TSA plates (Bio-Merieux, Marcy L'Etoile, France) and TSA-erythromycin, with no significant differences being observed between these media. The average numbers of bacteria per organ were calculated from the results obtained from the infected mice.

**Nucleotide sequence accession numbers.** The *plcA* nucleotide sequences were assigned the following GenBank accession numbers: strain BO43, AY367414; strain CNL895807, AY367410; strain CNL895795, AY367411; strain 416, AY367412; and strain 417, AY367413. The *plcB* sequence from strain 454 was assigned accession number AY787787.

## RESULTS

**Characterization of the low-virulence *L. monocytogenes* strains after cell interactions.** In contrast to virulent strains, 24 out of the 26 low-virulence strains formed no or only a few plaques (Table 1). In order to identify which step of the cell cycle had been altered, we compared their abilities to associate and enter human enterocytes.

The virulent and low-virulence strains had similar degrees of adhesion-invasion (i.e., including both adhered and intracellular bacteria) with HT-29 cells (Fig. 1a). On the other hand, the invasion assay, which assessed intracellular bacteria, displayed major differences (Fig. 1b). The penetration rates of 11 out of the 26 low-virulence strains were close to that of the noninvasive *L. innocua* strain (0.0007%). Therefore, impaired invasion rather than impaired adhesion seemed to account for the reduction of virulence.

**Characterization of the low-virulence field *L. monocytogenes* strains by enzyme activities.** Since the PC-PLC, PI-PLC, and LLO proteins were shown to be involved in the cell infection cycle, we measured their activities in *L. monocytogenes* strains.

According to Geoffroy et al., the PC-PLC activity was considered positive when it was above 1 U/ml (13). All the virulent strains had PC-PLC activities of 3 U/ml or more (Fig. 2a). However, only 12 of the 26 low-virulence strains had a significant PC-PLC activity. The other low-virulence strains (14 out of 26) had no significant PC-PLC activity, like the noninvasive *L. innocua* species.

The PI-PLC activity of bacteria grown in BHI broth supplemented with activated charcoal was also measured, according to the method of Goldfine and Knob (14). All the virulent strains had PI-PLC activities of over 1 pmol of inositol-monophosphate per ml per min (Fig. 2b). This threshold was set in accordance with the results obtained with the *L. innocua* strain. On the other hand, only 10 out of the 26 low-virulence strains had an enzyme activity of over 1 pmol/ml. Moreover, 11 out of the 16 low-virulence strains without PI-PLC activity had no detectable PC-PLC activity.

Under our conditions, all the low-virulence strains had a hemolytic activity of at least 1 hemolytic unit (Fig. 2c). Variance analysis shows that the virulent strains used exhibited higher hemolytic activity than the low-virulence strains. Fourteen out of 26 low-virulence strains had low hemolytic activity (less than 8 hemolytic units), while only 4 out of 13 virulent strains had low hemolytic activity. The virulent strain SO93 had no hemolytic activity with sheep red blood cells but hemolysed horse red blood cells (data not shown). These differential hemolytic responses were also observed by Van der Kelen and Lindsay (41). In accordance with reports showing that, when significant, the hemolytic titer is not related to the level of virulence, we did not take this factor into account for the classification of the low-virulence strains (22).

**Classification of the low-virulence *L. monocytogenes* strains into four groups.** Only four of the six criteria studied were used for the statistical analysis. Hemolytic activity was not taken into account because all the strains were hemolytic. Similarly, we did not use adhesion because all the *L. monocytogenes* strains analyzed adhered at similar levels, regardless of their virulence. We used an ascendant clustering hierarchical technique to classify the values of the four factors (cell invasion, plaque formation, and the activities of the two phospholipases C), thereby dividing the low-virulence strains into four groups (Fig. 3). Group I included strains that did not enter cells, formed no plaques, and had no phospholipase activity; group II included strains that entered cells but did not form plaques and expressed only PI-PLC; and group III included strains that entered cells but did not form plaques and expressed only PC-PLC. In contrast to groups I, II, and III, strains in group IV were indistinguishable from virulent strains on the basis of these in vitro characteristics: they entered cells, formed at least some plaques, and expressed all the phospholipase activities.

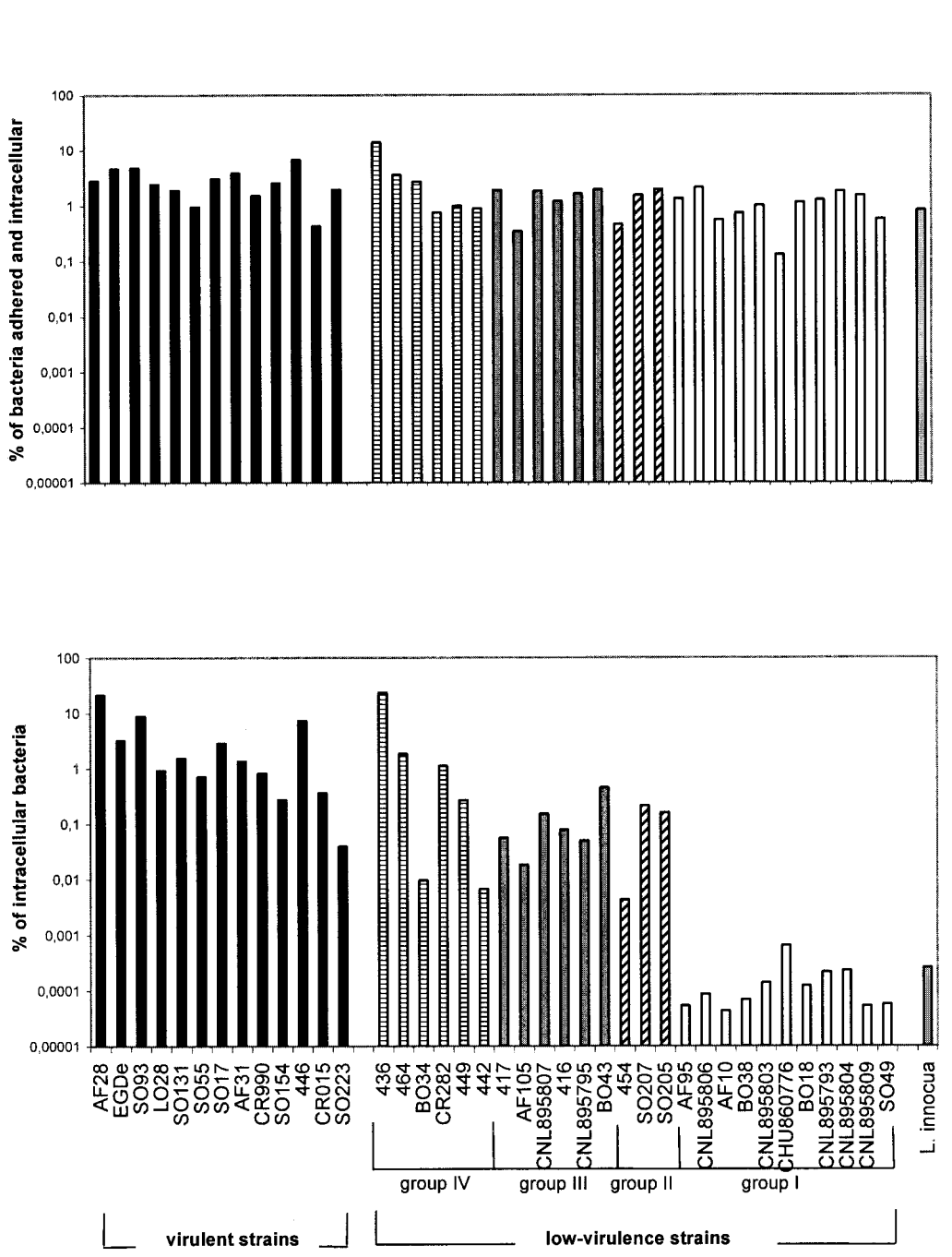


FIG. 1. Virulent and low-virulence strains compared after different cell invasion assays on HT-29 cell monolayers.

**Sequencing of the *plcA*, *plcB*, and *prfA* genes of the low-virulence *L. monocytogenes* strains.** In order to investigate whether the phenotypes observed could be due to genotypic mutations, we sequenced the *plcB* and *plcA* genes, encoding PC-PLC and PI-PLC, respectively, whose activities were not significant in group II and III strains. For group I strains, the *prfA* gene, encoding the transcriptional regulator of the virulence factors, was sequenced. The genes were compared according to their serovars to those of the virulent EGDe strain of serovar 1/2a (GenBank accession number AL591974) or to those of strain CLIP 80459 of serovar 4b (P. Glaser, personal communication).

For group II, strain 454 exhibited six mutations in the *plcB* gene, which led to four substitutions in the PC-PLC protein: Asp61Glu, Leu183Phe, Gln216Lys, and Ala223Val (Table 3). This strain exhibited a very low PC-PLC activity. While the SO205 and SO207 strains exhibited no PC-PLC activity, no difference could be detected in the *plcB* gene compared to that of the reference strain.

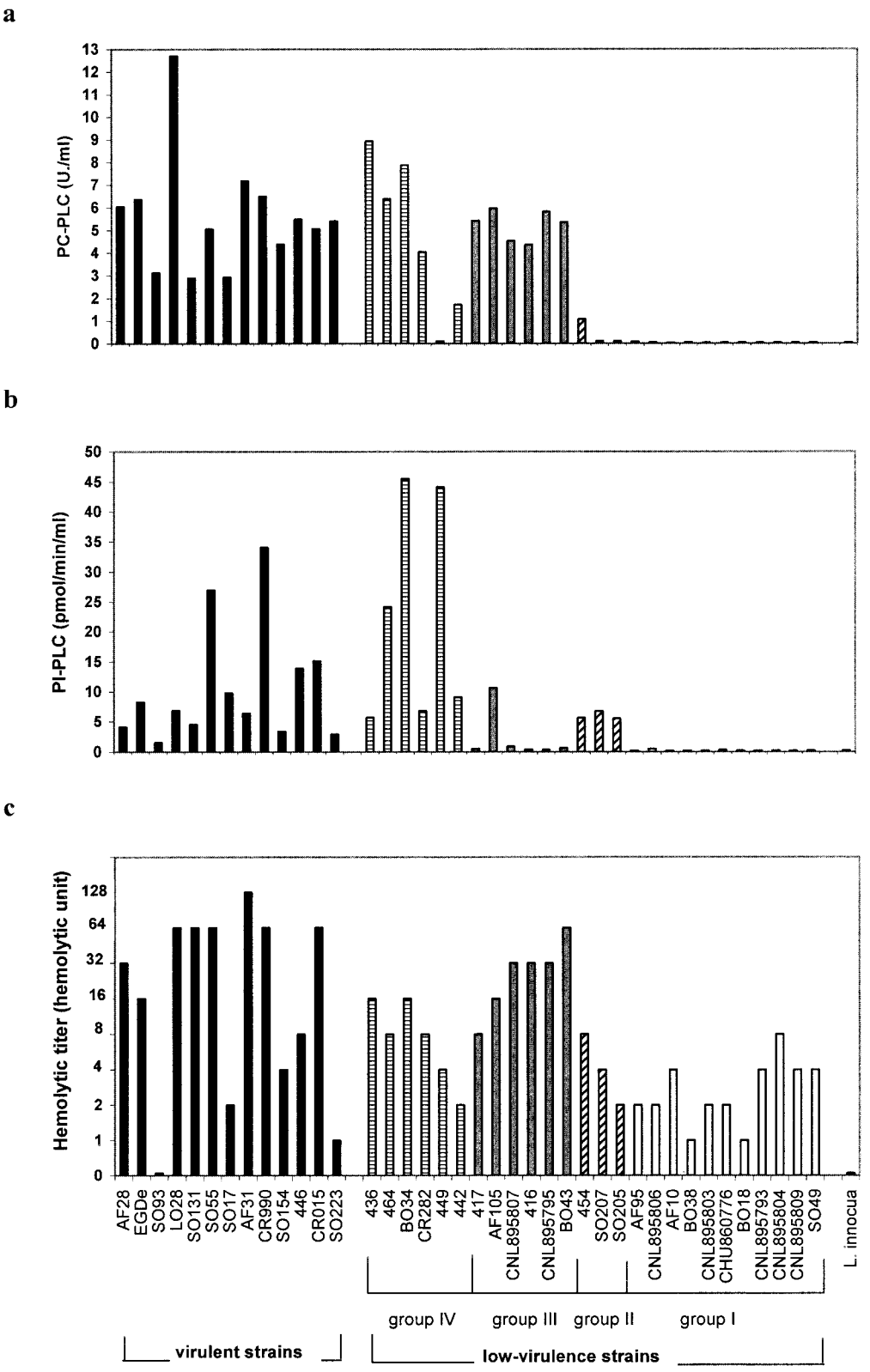


FIG. 2. Virulence-associated enzyme activities of virulent and low-virulence strains.

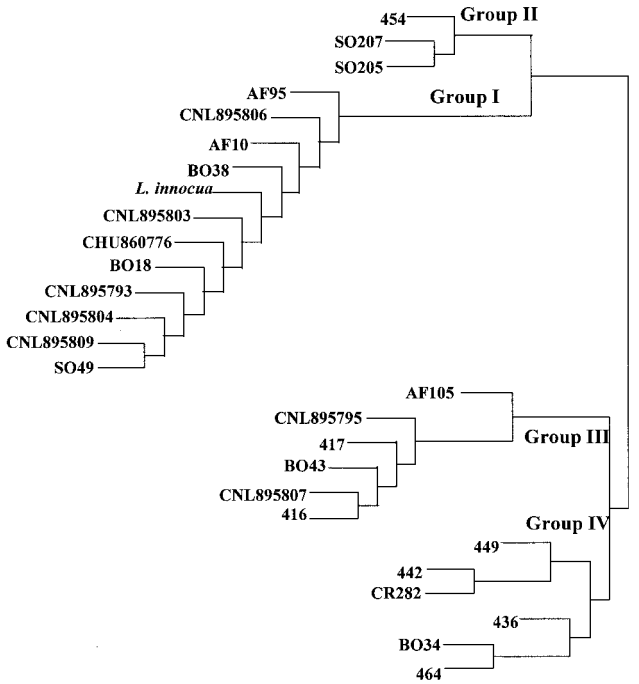


FIG. 3. Classification of the low-virulence *L. monocytogenes* strains. For this analysis we used an ascendant model-based agglomerative hierarchical clustering technique based on the results of cellular entry, plaque formation, and the two phospholipase C activities.

For group III, five strains out of six (CNL 895807, CNL895795, 416, 417, and BO43) exhibited the same 12 mutations in the *plcA* gene, which led to three substitutions in the PI-PLC protein: Ile17Val, Phe119Tyr, and Thr262Ala (Table 3). No PI-PLC activity could be detected for these strains. For the AF105 strain, no difference could be detected with the *plcA* gene of the virulent EGDe strain. This strain expressed PI-PLC activity.

The *prfA* gene sequences of the 11 group I strains showed mutations. The DNA sequences of three low-virulence strains (AF95, BO18, and BO38) had 7 nucleotides inserted after codon 171. This insertion changed the reading frame from codon 174 and introduced an early termination codon at position 184 (Table 3). The potentially shorter PrfA protein (PrfAΔ174–237) was analyzed by Western blotting with specific anti-PrfA antibodies. The three strains (AF95, BO18, and BO38) harboring the PrfAΔ174–237 mutation gave a band migrating at 22 kDa, which was thus smaller than the band corresponding to the wild-type PrfA protein encoded by the EGDe strain (28 kDa) (data not shown). This was in accordance with the molecular weight of the putative truncated PrfA protein. We detected an A-to-C transition at the first position of codon 220 in the other eight group I low-virulence strains (AF10, CHU860776, CNL895793, CNL895803, CNL895804, CNL895806, CNL895809, and SO49). This nucleotide replacement led to a Lys220Thr substitution in PrfA (PrfAK220T) (Table 3). Therefore, only two mutations were found in the *prfA* genes of these 11 low-virulence field strains of *L. monocytogenes*, which were unrelated in origin and isolation date and belonged to three different rRNA gene restriction patterns (data not shown).

**Effect of the mutations in the *prfA* genes of the *L. monocytogenes* group I strains.** The influence of the two mutations on PrfA activity was demonstrated by introducing the mutated *prfA* gene encoding the truncated PrfA protein (PrfAΔ174-237) of strains BO18 and AF95 or the mutated PrfA protein (PrfA220KT) of strains CNL895806 and SO49 in *trans* in an EGDe strain lacking the *prfA* gene (EGD Δ*prfA*). The EGD Δ*prfA* strain transformed with one of the mutated *prfA* genes had the same low hemolytic activity as the parent EGD Δ*prfA* strain and the EGD Δ*prfA* strain carrying the pP1 vector without an insert. On the other hand, the hemolytic titer of the EGD Δ*prfA* strain carrying the wild-type *prfA* gene in *trans* was eight times higher than that of the parent strain (data not shown). Similar results were obtained for PC-PLC activity: no activity was detected for EGD Δ*prfA* and the derivative strains carrying only the pP1 vector or the recombinant plasmid with the mutated *prfA*. PC-PLC activity was recorded only with the EGD Δ*prfA* strain carrying the wild-type *prfA* gene in *trans* (data not shown). Thus, our results demonstrate that *trans*-complementation of EGD Δ*prfA* by the genes encoding PrfAΔ174–237 or PrfAK220T did not restore the PC-PLC or hemolytic activities, unlike complementation with the wild-type *prfA* gene, demonstrating that these mutations inactivate PrfA.

In order to demonstrate that these mutations were sufficient to explain the low virulence, the BO18 and AF95 strains, producing truncated PrfA protein (PrfAΔ174–237), and the CNL895806 and SO49 strains, producing mutated PrfA protein (PrfAK220T), were complemented with the wild-type *prfA*

TABLE 3. Substitutions found in the PrfA, PC-PLC, and PI-PLC proteins

| Strain | Substitution(s) in: | | |
|---|---|---|---|
| | PrfA | PC-PLC | PI-PLC |
| Group I | | | |
| CHU860776 | K220T | | |
| CNL895793 | K220T | | |
| CNL895803 | K220T | | |
| CNL895804 | K220T | | |
| CNL895806 | K220T | | |
| CNL895809 | K220T | | |
| SO49 | K220T | | |
| AF10 | K220T | | |
| BO18 | Δ174–237 | | |
| BO38 | Δ174–237 | | |
| AF95 | Δ174–237 | | |
| Group II | | | |
| SO205 | | None | |
| SO207 | | None | |
| 454 | | D61E, L183F, Q216K, A223V | |
| Group III | | | |
| AF105 | | | None |
| CNL895807 | | | I17V, F119Y, T262A |
| CNL895795 | | | I17V, F119Y, T262A |
| 416 | | | I17V, F119Y, T262A |
| 417 | | | I17V, F119Y, T262A |
| BO43 | | | I17V, F119Y, T262A |

TABLE 4. Virulence assays performed with wild-type and *trans*-complemented *L. monocytogenes* strains

| Strain | Plasmid inserted[a] | Virulence (mean ± SD) by: | | |
|---|---|---|---|---|
| | | PF assay[b] | s.c. test (spleens)[c] | *I/T*[d] |
| EGDe | np | 6.35 ± 0.12 | 5.54 ± 0.46 | 5/5 |
| EGD Δ*prfA* | np | 0.00 | 0 | 0/5 |
| | pP1[f] | 0.00 | 0 | 0/5 |
| | $pPrfA_{EGDe}$ | 0.00[e] | 0 | 0/5 |
| AF95 | np | 0.00 | 0 | 0/5 |
| | pP1 | 0.00 | 0 | 0/5 |
| | $pPrfA_{EGDe}$ | 6.01 ± 0.14 | 3.07 ± 1.25 | 5/5 |
| BO18 | np | 0.00 | 0 | 0/5 |
| | pP1 | 0.00 | 0 | 0/5 |
| | $pPrfA_{EGDe}$ | 5.86 ± 0.09 | 3.50 ± 0.55 | 5/5 |
| CNL895806 | np | 0.00 | 0 | 0/5 |
| | pP1 | 0.00 | 0 | 0/5 |
| | $pPrfA_{EGDe}$ | 6.03 ± 0.02 | 2.06 ± 0.91 | 5/5 |
| SO49 | np | 0.00 | 0 | 0/5 |
| | pP1 | 0.00 | 0 | 0/5 |
| | $pPrfA_{EGDe}$ | 5.65 ± 0.11 | 4.17 ± 0.39 | 5/5 |

[a] np, no plasmid; pP1, pP1 plasmid without insert; $pPrfA_{EGDe}$, plasmid carrying the wild-type *prfA* gene.
[b] Log numbers of plaques per $10^7$ CFU deposited. Values are from two independent experiments performed in duplicate.
[c] Log numbers of *Listeria* recovered in the spleens 3 days after s.c. injection into the left hind footpads of immunocompetent Swiss mice with $10^4$ CFU in 50 μl. Values are from infected mice.
[d] Ratio of infected mice to inoculated mice in s.c. test.
[e] HT-29 cell monolayers were damaged when they were infected with at least $10^7$ CFU deposited per well.
[f] pP1 vector previously described by Dramsi et al. (10).

gene from the EGDe strain inserted in *trans*. The transformed strains were tested in the PF assay and after subcutaneous inoculation of mice. In contrast to the four low-virulence parent strains, which did not enter HT-29 cells and thus formed no plaques, the *trans*-complemented strains formed the same number of plaques as the virulent EGDe strain (Table 4). Surprisingly, the EGD Δ*prfA* strain transformed with the wild-type *prfA* gene did not form plaques but destroyed the HT-29 cell monolayers. Likewise, this strain did not recover its virulence in vivo. All the low-virulence strains *trans*-complemented with the wild-type *prfA* gene were able to infect all the mice inoculated, whereas the parental low-virulence strains were avirulent in this model. Our data therefore suggest that the addition of wild-type *prfA* in *trans* was sufficient to restore the virulence of low-virulence strains carrying a mutated *prfA* gene and therefore that any other mutation would not significantly alter their virulence.

## DISCUSSION

Several reports have described nonpathogenic or weakly pathogenic strains of *L. monocytogenes*, but little is known about what determines low virulence. To date, the lack of hemolytic activity has been shown to be linked to the nonvirulence of field strains (8). In addition, Jacquet et al. have statistically demonstrated the critical role of internalin in the pathogenesis of human listeriosis and have suggested that risk should be evaluated not only on the basis of levels of bacterial contamination but also on the basis of the functionality of internalin (19). We previously identified 26 low-virulence field strains, and the present study was designed to identify virulence factors or cell invasion mechanisms that had been altered in these low-virulence strains. We thus analyzed the different stages of cell infection and also the characteristic enzymatic activities of the virulence genes. Cluster analysis assigned the low-virulence *L. monocytogenes* strains to four groups corresponding to similar phenotypes, using four of the six criteria analyzed (invasion, plaque formation, and PC-PLC and PI-PLC activities). Based on the phenotypic groups identified in this way, we hypothesized that specific genes could be responsible for the observed differences. We therefore sequenced these specific genes to identify mutations that could have altered the functions of the genes.

Interestingly, a link was found between the groups and the virulence of the strains previously described by Roche et al. (32, 33). Ten of the 11 group I strains were avirulent and not lethal in mice, even after the s.c. injection of $10^9$ CFU. Only 1 of the 11 strains was hypovirulent and not lethal in mice. Similarly, four of the six group IV strains were hypovirulent, and three had 50% lethal doses of close to $10^8$ to $10^9$ CFU. The two other strains were avirulent.

The characteristics of group IV strains, all belonging to serogroup 4 and serovar 7, were similar to those of the virulent strains in terms of their cell infection phenotype and phospholipases C activities. The main differences between these strains and virulent strains were the frequency of spleen infection after s.c. inoculation and the course of infection in vivo (32). This is supported by the fact that two strains belonging to this group (strains 464 and CR282) originated from human cases.

The group II (three strains) and III (six strains) strains were phenotypically very similar. They all had poor in vivo colonization capacities and entered cells but did not form plaques. Group II strains had very low PC-PLC activities, while group III strains had very low PI-PLC activities. This low activity could be related to the substitutions found in the *plcA* gene for five out of six group III strains. For the group II strains, it is premature to make any assumptions because only one out of three group II strains showed mutations in the *plcB* gene. However, Smith and Portnoy have demonstrated that the lack of one phospholipase only slightly decreases virulence (37). Thus, it is more than likely that the low virulence of these strains is related to other mutations. We are currently analyzing the role of substitutions in the activity of these virulence-related enzymes.

The 11 group I strains did not enter host cells and thus formed no plaques. They had little or no phospholipase C activity and were weakly hemolytic. As these bacterial functions are controlled by PrfA, we analyzed the sequence of the *prfA* gene, encoding the transcriptional regulator of *L. monocytogenes* virulence genes. Only two naturally occurring mutations were found among the 11 low-virulence *L. monocytogenes* strains, even though they were unrelated epidemiologically and belonged to three different rRNA gene restriction patterns. Three strains had seven nucleotides inserted into their *prfA* gene, introducing an early termination codon that resulted in a truncated protein (PrfAΔ174–237) as demonstrated by Western blotting. Eight strains had the same nucleotide substitu-

tion, leading to a Lys220Thr substitution (PrfAK220T). The two mutation types prevented the activation of virulence genes, since insertion of a plasmid carrying the mutated *prfA* into the EGD Δ*prfA* strain did not restore hemolytic or PC-PLC activity, unlike insertion of wild-type *prfA* gene. These naturally occurring mutations explain the low virulence of the 11 field *L. monocytogenes* strains, because these strains recovered their ability to form plaques in HT-29 cell monolayers when they were *trans*-complemented with the wild-type *prfA* gene from the EGDe strain. The *trans*-complemented strains also recovered their virulence in mice after s.c. inoculation. They all infected 100% of inoculated mice, in contrast to the case when the wild-type *prfA* gene was inserted in *trans* into the EGD Δ*prfA* strain, which led to cytotoxic effects without plaque formation. This cytotoxicity could be due to the strong constitutive promoter that controls the transcription of *prfA* on the plasmid. Indeed, overexpression of the *prfA* gene could lead to synthesis of large amounts of all the virulence factors in the EGDe strain, fast destruction of infected cells, and thus damage to cell monolayers. Moreover, no mice were infected by this strain. In low-virulence strains, on the other hand, the presence of inactive PrfA may lead to the formation of less efficient heterodimers.

To our knowledge, this is the first time that the truncated PrfA protein has been described for field strains of *L. monocytogenes*, although spontaneous deletions in the *prfA* gene have been described for reference culture collection strains (27). The truncated PrfA protein in our strains led to the loss of both the helix-turn-helix DNA-binding motif (amino acids 171 to 191) mediating the interaction of PrfA with the PrfA box in target promoters (35) and the putative leucine zipper motif (amino acids 193 to 237) (24). The second mutation found in the other eight strains was the replacement of the lysine at position 220 by a threonine. It occurred just in front of the leucine at position 221, one of the characteristic amino acids of the putative leucine zipper motif that could predict a dimerization domain (24). Several experiments are in progress to understand the role of the K220T substitution in the formation of homodimers and in the binding to the PrfA boxes.

Overall, the sequencing of virulence genes in low-virulence strains has shown a link between the phenotypic classification and genotypic modifications, at least for groups I and III. These widespread mutations could reflect an evolution of the *L. monocytogenes* strains as observed with *L. innocua*, which lost the virulence gene cluster (43). Indeed, 30% of the 26 low-virulence field strains detected in our laboratory had the same nucleotide replacement in *prfA* (PrfAK220T), and 12% had the same 7-nucleotide insertion in the 3′ region of *prfA* (PrfAΔ174–237). In addition, 19% of the strains (group III) showed the same 12 mutations in the *plcA* gene. The common evolution of some low-virulence strains is reinforced by analysis of the DNA macrorestriction profiles, which are different from those of the 623 virulent *L. monocytogenes* strains tested (A. Kerouanton, personal communication). It now seems important to investigate whether these substitutions are due to a common ancestry or to horizontal transfer, as is the case for the natural atypical *L. innocua* (21).

Overall, our results show that some *L. monocytogenes* strains have genetic mutations based on their phenotypes that could be predicted to a certain extent. It should therefore be possible to identify the amino acids relevant for virulence protein activity. Further work could involve carrying out a multiplex PCR assay for the screening of the low-virulence strains, since the genetic mutations appear to be specific to each group.

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